

Evans, et al.
U.S.S.N. 09/249,543
Filed: February 19, 1999
Page 2

Sub D2
C2 2. The method of claim 1, wherein said first target protein of step (a) is generated from a first plasmid which further comprises at least one nucleic acid sequence that encodes at least one first intein having N-terminal cleavage activity and said second target protein of step (b) is generated from a second plasmid which further comprises at least one nucleic acid sequence that encodes at least one second intein having C-terminal activity.

C3 3. The method of claim 2, wherein said at least one first intein comprises a first modified *Mth* RIR1 intein and wherein said at least one second intein comprises a second modified *Mth* RIR1 intein.

31. A method according to claim 2, wherein at least one first or at least one second intein may be an unmodified or a modified form of a naturally occurring intein.

C4 32. A method according to claim 1, wherein the C-terminal thioester of step (a) is formed in the presence of a thiol reagent.

33. The method of claim 32, the thiol reagent is 2-mercaptoethanesulfonic acid.

Sub D8
34. The method of claim 3 further comprising:
replacing in the first intein, a terminal proline residue with an alanine residue, the alanine residue having an N-terminal position with respect to a first amino-acid of the intein.

Evans, et al.
U.S.S.N. 09/249,543
Filed: February 19, 1999
Page 3

35. The method of claim 3, further comprising:
replacing a C-terminal asparagine or cysteine of the intein with an
alanine.

36. The method of claim 2, wherein the first and second plasmids
are capable of expression in at least one cell type selected from the
group consisting of a bacterial, yeast, plant, insect and mammalian cell
type.

C4 37. The method of claim 8, wherein step (b) further comprises
cleaving of an intein controllably, or by induction using a nucleophilic
compound.

38. The method of claim 37, wherein the nucleophilic compound is
a thiol reagent.

39. The method of claim 37, wherein controlling cleavage of the
intein includes modulating temperature, pH, salt, chaotropic agents, or
any combinations thereof.

Jul 09 40. A method for ligating a first protein target to a second target
protein, comprising:

- (a) applying means for generating fusion proteins of the first
protein and at least one first intein and a second protein and
at least one second intein where the first intein and the
second intein may be the same or different;

Evans, et al.
U.S.S.N. 09/249,543
Filed: February 19, 1999
Page 4

- (b) applying means for cleaving the first and the second fusion protein so as to provide a C-terminal thioester on one target protein and a specified N-terminal on the second target protein; and
- (c) applying means for permitting the first target protein to ligate to the second target protein.

C4 41. A method according to claim 40, wherein step (b) further comprises applying means for separating the first and second target proteins from the cleaved inteins.

42. A method for obtaining a protein product formed from two target proteins, said method comprising the steps of:

- See D9 cont
- (a) generating a first target protein fused to at least one first intein and a second target protein fused to at least one second intein, wherein the first intein may be the same or different from the second intein;
 - (b) cleaving the first target protein from at least one first intein so as to form a C-terminal thioester; and cleaving the second target protein from at least one second intein so as to provide a specified N-terminal; and
 - (c) ligating the first target protein with the second target protein to form the protein product.

43. The method of claim 42, wherein the first target protein of step (a) is generated from a first plasmid which further comprises at least one nucleic acid sequence that encodes the at least one first intein and

Evans, et al.
U.S.S.N. 09/249,543
Filed: February 19, 1999
Page 5

said second target protein of step (a) is expressed by a second plasmid which further comprises at least one nucleic acid sequence that encodes the at least one second intein.

44. The method of claim 43, wherein the first intein comprises a first modified *Mth* RIR1 intein and wherein the second intein comprises a second modified *Mth* RIR1 intein.

C4 45. The method of claim 44, wherein the first modified *Mth* RIR1 intein is selected from the group consisting of a Pro⁻¹ to Gly mutant intein, and a Pro⁻¹ to Asn¹³⁴ to Gly-Ala mutant intein, and wherein said modified *Mth* RIR1 intein is selected from the group consisting of a Pro⁻¹ to Cys¹ to Gly-Ser mutant intein and a Pro⁻¹-Cys¹ to Gly-Ala mutant intein.

46. The method of claim 43, wherein the first plasmid is selected from the group consisting of pMRB8A, pMRB8G1 and pMRB9GA and pBRL-A and wherein the second plasmid is selected from the group consisting of PMRB9GS, pMRB9GA and pBRL-A.

47. The method of claim 44, wherein the first target protein of step (a) is generated by a thiol reagent-induced cleavage product of said first modified *Mth* RIR1 intein and said second target protein of step (a) is generated by temperature and/or pH induced cleavage of said second modified *Mth* RIR1 intein.

48. The method of claim 43, wherein the specified N-terminal comprises a cysteine.

Evans, et al.
U.S.S.N. 09/249,543
Filed: February 19, 1999
Page 6

49. A method according to claim 43, wherein at least one first or at least one second intein may be an unmodified or a modified form of a naturally occurring intein.

50. A method according to claim 42, wherein the C-terminal thioester of step (b) is formed in the presence of a thiol reagent.

C4 51. The method of claim 50, the thiol reagent is 2-mercaptoethanesulfonic acid.

52. The method of claim 44, further comprising:
replacing in the first intein, a terminal proline residue with an alanine residue, the alanine residue having an N-terminal position with respect to a first amino acid of the intein.

53. The method of claim 44, further comprising:
replacing a C-terminal asparagine or cysteine of the intein by an alanine.

54. The method of claim 43, wherein the first and second plasmids are capable of expression in at least one cell type selected from the group consisting of a bacterial, plant, insect and mammalian cell type.

55. The method of claim 49, wherein step (b) further comprises:
cleaving of an intein controllably, or by induction using a nucleophilic compound.

Evans, et al.
U.S.S.N. 09/249,543
Filed: February 19, 1999
Page 7

56. The method of claim 55, wherein the nucleophilic compound is a thiol reagents.

57. The method of claim 55, wherein controlling cleavage of the intein includes modulating temperature, pH, salt, chaotropic agents, or any combinations thereof.

58. A method for generating a protein or peptide having a specified N-terminal amino acid, comprising:

C4 obtaining a nucleic acid encoding the protein or peptide having an intein coding sequence adjacent to a specified amino acid codon of the target protein;

causing the nucleic acid product to be expressed; and

cleaving the intein from the expressed nucleic acid product so as to generate the protein or peptide with the specified N-terminal amino acid.

59. A method for obtaining an expressed protein with a C-terminal thioester, comprising:

- (a) obtaining the expressed precursor protein, the precursor having an intein; and
- (b) reacting the precursor protein with a thiol reagent so as (i) to remove the cleavage element and (ii) to obtain the expressed protein with the C-terminal thioester.

60. The method of claim 59, wherein the intein is an *Mth* RIR1 intein.